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UNITED KINGDOM 008.06687001

4. Title of the invention

STARCH BIOSYNTHESIS

5. Name of your agent (if you have one)

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STARCH BIOSYNTHESIS

The present invention relates to starch biosynthesis in plants and more particularly to the modulation of such biosynthesis for the purposes of, for example, producing modified starches and/or increasing starch yield. The invention also relates to methods of modulating such biosynthesis and also to genetically modified plants and plant cell lines in which starch biosynthesis is modulated.

Starch biosynthesis in plants is the major determinant of yield in agricultural production of cereals. On a world basis cereals contribute 50% of total dietary energy supplies, and up to 75% of the human daily calorific intake is starch. Starch also has a broad range of industrial applications (e.g. in manufacture of paper, paint and adhesives). Variation in the chemical structure of starch, determined by the ratio of amylose to amylopectin and the degree of branching in amylopectin in the starch polymer, can significantly alter the properties of starch and the regulation of such structure is highly desirable for optimising the industrial application in which starch is being used.

In plant storage tissues the biosynthesis of starch occurs in specialised cellular organelles called amyloplasts. The immediate soluble substrate for starch synthesis is the sugar nucleotide ADP glucose (ADPG). Amyloplasts must import the carbon required for starch synthesis and we have previously demonstrated that amyloplasts of wheat endosperm are capable of synthesising starch from either exogenous sugar (hexose) phosphates and ATP, or by direct uptake of ADPG. Recent evidence has demonstrated that in cereal endosperm the vast proportion of the enzyme which synthesises ADPG is located in the cytoplasm (outside the amyloplast).

We have performed studies with amyloplasts which show that ADPG supports rates of starch synthesis 30-fold greater than from hexose-phosphate uptake. Furthermore we have found during the active period of grain filling nearly all of the

enzyme activity responsible for the synthesis of ADPG resides in the cytoplasm. This lead us to realise that the major source of carbon taken up by the amyloplast for starch synthesis is ADPG.

As synthesis of ADPG is located in the cytoplasm, the transport protein responsible for uptake of ADPG into amyloplasts must therefore play a pivotal role in regulating starch synthesis. The ADPG transporter influences not only starch yield, but also quality (a commercially important aspect of this raw material) since the starch synthases involved in amylose and amylopectin synthesis have different affinities for ADPG.

The present invention is founded on our identification of a protein which is integral to the membrane of amyloplasts (e.g. amyloplasts from developing wheat endosperm) and which, we have established, is the ADPG transporter. The protein is the most abundant membrane protein in amyloplasts and may be obtained by the procedure of Example 1 (see below).

The ADPG transporter protein may be characterised by the following features:

- (a) an amino acid sequence at the N-terminus of the protein:

SMPLNAAVKM;

- (b) three internal amino acid sequences:

GAXXXETAWACGXA

PYNEAYQOXG

NFRYTNFAXF

(wherein X indicates any amino acid and the other letters represent the conventional single letter code for amino acids); and

(c) a molecular weight of about 38 kDa.

A protein comprising any of the amino acid sequences listed under (a) or (b) above represents a new protein and according to a first aspect of the present invention there is provided an ADP-Glucose transporter protein, or a modification or fragment thereof capable of ADP-Glucose transport activity comprising at least one amino acid sequence selected from the group of:

- (i) SMPLNAAVKM;
- (ii) GAXXXETAWACGXA;
- (iii) PYNEAYQOXG; and
- (iv) NFRYTNFAXF.

The protein of the first aspect of the invention preferably comprises two or more of the amino acid sequences (i) - (iv) and most preferably all such sequences.

The protein preferably has a molecular weight of approximately 38 kDa.

The protein may be isolated from membranes of amyloplasts from cereal endosperm (e.g. spring wheat endosperm).

By "modifying" or "controlling" the amount and / or activity of a protein in accordance with the first aspect of the invention in a starch producing plant (as compared to the amount which is present in a wild-type plant) it is possible to modulate starch production in various desirable ways. Thus according to a second aspect of the present invention there is provided a method of regulating starch production in plants or

plant cells comprising modulating the activity of the protein according to the first aspect of the invention.

According to a first embodiment of this method the activity of the ADPG transporter may be decreased in amyloplast membranes of plant cells. A decrease in the amount of protein will favour production of starch with a higher proportion of amylopectin resulting in the production of "waxy" starches which can be exploited as thickening agents in food and coatings.

A decrease in activity can be achieved by exogenous addition of an agent which suppresses transporter activity (i.e. an ADPG transporter antagonist or a neutralising species raised against the transporter). Alternatively an agent may be used which increases the rate of breakdown of active transporter or one which decreases expression of the protein (e.g. protein synthesis inhibitors, inhibitors of post-translational modification, ribosymes, antisense RNA or DNA, inhibitory transcription factors etc).

According to a second embodiment of this method the activity of the ADPG transporter is increased in amyloplast membranes of plant cells. This favours increased starch yield and will favour production of starch with a higher proportion of amylose. Increasing amylose content increases the viscosity and gel strength of starch pastes. Such starches can be exploited in the production of biodegradable plastics. High amylose content also influences gelatinization and retrogradation properties (crystallisation of cooling starch components following heating) of starches. Since retrogradation of amylopectin is a factor influence the rate at which baked foods go stale, we have found that baked foods comprising starches with an increased proportion of amylose goes stale less quickly and therefore have longer shelf lives. For instance, bread comprising starches containing an increased proportion of amylose go stale less quickly than bread containing unmodified starch.

An increase in activity can be achieved by exogenous addition of an agent which increases activity of the transporter according to the first aspect of the invention (i.e. an ADPG transporter agonist or an activating species). Alternatively an agent may be used which decreases the rate of breakdown of active transporter.

The activity may also be promoted by increasing the amount of transporter in the amyloplast membrane. This may be achieved by application of the protein to a plant or plant cell in a form in which the protein will be taken up and incorporated into the membranes of the starch producing organelles of the plant. However it is preferred that the amount of transporter in the amyloplast membrane is increased by promoting expression of the protein. This may be achieved by increasing the activity of transcription factors which promote gene expression (e.g. by administering to a plant cell the transcription factors *per se* or by administering phytohormones or other agents which promote the activity of these transcription factors). It is most preferred to increase ADPG transporter expression by genetically manipulating a plant cell.

Starch production is preferably regulated according to the first or second embodiments of the method of the invention by genetically manipulating plant material. Such manipulation requires the use of a DNA molecule which codes for protein capable of modulating ADPG transporter activity. Thus according to a third aspect of the invention there is provided a DNA molecule encoding for a protein which directly or indirectly modulates ADP-glucose transporter protein activity, said DNA molecule being capable of being transcribed to lead to the expression of said protein.

Said protein directly or indirectly has activity for modulating ADP-Glucose transporter protein activity such that starch production from a plant cell is regulated. By "directly" we mean that the protein coded by the DNA molecule *per se* has the required activity for regulating starch production. By "indirectly" we mean that the protein coded by the DNA molecule undergoes or mediates (e.g. as an enzyme) at

least one further reaction to provide an agent effective for regulating starch production.

A preferred DNA molecule according to the third aspect of the invention codes for a protein of the first aspect of the invention. Using such DNA molecules, it is possible to produce genetically modified plant material having an enhanced capability for the production of the protein of the first aspect of the invention so as to modulate starch production according to the second embodiment of the second aspect of the invention. These preferred DNA molecules are particularly useful for transforming a plant cell such that it will contain multiple copies of genes encoding proteins of the first aspect of the invention. Expression of the protein from such multiple copies may give rise to increased copies of proteins with transporter activity greater than is found in unmanipulated plant cells.

It will be appreciated that the base sequence of DNA molecules according to the third aspect of the invention may exhibit some base variability but still code for the same, or a functionally equivalent protein. For instance, DNA molecules which encode for the ADP-Glucose transporter or a modification or fragment of such a protein which retains ADPG transport activity, may have base changes which would not alter the amino acid sequence of the transporter expressed from the DNA molecule (e.g. due to redundancy in the genetic code). Variants of the DNA molecule may even encode proteins with amino acid changes which do not alter the transporter's function.

It is preferred that DNA molecules according to the third aspect of the invention are propagated within a suitable DNA vector to form a recombinant vector. The vector may for example be a plasmid, cosmid or virally based (e.g. Gemini virus vectors). Such recombinant vectors are of utility when replicating the DNA molecule. Furthermore recombinant vectors or derivatives thereof are highly useful for transforming plant cells or protoplasts.

The recombinant vectors will frequently include one or more selectable markers to enable selection of cells transformed with the DNA vector and, preferably, to enable selection of cells harboring the recombinant vectors that incorporate the DNA molecule of the third aspect of the invention. Examples of such selectable markers include genes conferring resistance to kanamycin, G418, phosphinothricin, ampicillin or neomycin.

Recombinant vectors may also include other functional elements. For instance, the recombinant vector may be designed such that the vector and DNA molecule integrates into the genome of a cell. In this case DNA sequences which favour targeted integration (e.g. by homologous recombination) are desirable. Alternatively recombinant vectors can be designed such that the vector will autonomously replicate in the cytosol of the cell. In this case, elements which induce DNA replication may be required in the recombinant vector. Preferred recombinant vectors which autonomously replicate in the cytosol of the cell will be capable of existing in a host cell in multicopies. Multiple copies of such vectors are useful for increasing expression of protein encoded by the DNA molecule of the third aspect of the invention above levels obtainable in normal wild-type plant cells (i.e. cells which have not been genetically modified).

The recombinant vector may also further comprise a promoter or regulator to control expression of the gene as required.

Preferred recombinant vectors may be formed using plasmids such as pBI 101, Bin 6, Bin 19 or pUC or derivatives of such vectors.

The recombinant vectors may be transferred to plant cells or protoplasts by, transformation, transfection, infection, microinjection, cell fusion, protoplast fusion or ballistic bombardment. For example, transfer may be by ballistic transformation with coated gold particles, agrobacterium mediated transformation, liposomes containing the DNA molecule, viral vectors (e.g. Gemini virus) by electroporation, by chemical

transformation (e.g. treating cells with calcium chloride and Polyethylene glycol favours DNA uptake) or by means of direct DNA uptake (e.g. endocytosis).

The DNA molecule according to the third aspect of the invention may be delivered, by the abovementioned methods, to a plant cell or protoplast to be transformed without it being incorporated in a vector.

Plant material genetically modified with a DNA molecule according to the third aspect of the invention represents a fourth aspect of the invention, according to which there is provided a plant cell transformed with the DNA molecule of the third aspect of the invention. Such transformed cells are particularly useful for regulating starch production according to the method of the second aspect of the invention.

Many different types of plant material may be treated according to the method of the second aspect of the invention or transformed according to the fourth aspect of the invention. The material may be a growing plant, plant tissue, a plant cell line, a seed or any other suitable source of plant material which is capable of growth. If desired plant tissue may be removed from the treated plant material for further cultivation. This tissue may be in the form of a growing shoot tip, a bud or a root tip from a plant. Such tissue may be cultivated in soil or may be grown *in vitro*. *In vitro* grown tissue may be grown under suitable conditions to give rise to a plant or alternatively the tissue may be grown to develop a plant cell line.

Plants to which the invention is applicable for modulating starch production include, but are not limited to, cereals (e.g. wheat, barley, oats and maize), rice, potatoes, sorghum or cassaba.

Cell lines which have been transformed according to the fourth aspect of the invention may be developed from such plants.

The plant material, plants and plant cell lines should be maintained or propagated in a suitable growth medium. When plants are grown in the ground, the environment may represent a suitable medium, although supplementation with phytohormones, vitamins and/or carbohydrate etc may be required. Plant material, plants and plant cell lines propagated *in vitro* require a suitable growth medium to sustain the cells.

Such plant material (plants, plant tissue or plant cell lines) may, for example, be produced by transforming plant cells (e.g. endosperm cells) and growing plants from said transformed cells. The plants may be grown and seeds collected following selfing of said plants. Alternatively progeny of the transformed cells may be collected by *in vitro* propagation of said transformed cells. The transformed cells may be propagated using essentially standard culturing techniques which are known in the art of plant science.

The invention is illustrated by the following non-limiting Example.

EXAMPLE 1

Protocol for Purification of the ADP glucose Transporter from Amyloplasts

Plant Material and Amyloplast Isolation

Spring wheat (*Triticum aestivum* L. Cv. Axona) was grown under the conditions described by Tetlow *et al.* (1993). Endosperm tissue was obtained from developing grains taken from the mid-ear region of the head 8-14 days after anthesis (the first appearance of anthers). The dissected endosperm (25-37g fresh weight) was used to prepare amyloplasts mechanically using the method described by Tetlow *et al.* (1994).

Isolation of Organelle Membranes

Purified amyloplasts were ruptured in a buffer containing 100mM tricine-NaOH (pH 7.8), 1mM EDTA, 1mM dithiothreitol, 1mM phenylmethylsulphonyl fluoride

(PMSF) and 100 μ M each of leupeptin, bestatin, pepstatin, 3,4-dichloroisocoumarin, chymostatin, 1,10-phenanthroline, phosphoramidon, and pefabloc (rupturing buffer). All subsequent procedures were performed at 2-4°C. The ruptured amyloplasts were centrifuged for 10 min at 10,000g to remove starch and debris. The supernatant was then centrifuged for 20 min at 100,000g to pellet the envelope membranes. The membrane pellet was resuspended in rupturing buffer containing 1.2M NaCl and left on ice for 30-60 min to remove extrinsic membrane proteins. The NaCl-washed membranes were pelleted (20 min, 100,000g) and the supernatant discarded, before being resuspended in rupturing buffer. The washed membranes were then finally pelleted (20 min, 100,000g) and rinsed three times in rupturing buffer before solubilization (below).

Membrane Solubilization and Protein Fractionation

The washed amyloplast envelope membranes were solubilized by resuspension in column running buffer (10mM Tricine-NaOH (pH 7.6), 0.2% (w/v) n-dodecylmaltoside, 1mM PMSF), followed by the addition of 40% (w/v) n-dodecylmaltoside, to give a final detergent concentration of 8%. After incubation on ice for 10 min the solubilized membranes were diluted with running buffer to give a final n-dodecylmaltoside concentration of 1.6%. The sample was centrifuged for 5 min at 13,000g to remove insoluble material and the supernatant loaded onto a 1cm³ HiTrap (Pharmacia) QTM column which had been pre-equilibrated in running buffer. After loading the sample, the column was washed in 5 column volumes of running buffer before running a stepped gradient of 0-2M NaCl (in running buffer). The 2x1cm³ fraction (containing ADP glucose transporter activity) eluting at 90mM NaCl was retained and diluted to 10cm³ in running buffer. The sample was then loaded on to a 1cm³ HiTrap (Pharmacia) BlueTM at a flow rate of 0.5cm³ per minute (column pre-equilibrated in running buffer). After loading the sample the column was washed in 5 column volumes of running buffer. The purified transporter was eluted from the column with 3x1cm³ of 30mM ATP in running buffer. The purified protein was concentrated down to about 0.5cm³ using AmiconTM microcentrifuge filters (10kDa molecular weight cut off).

Reconstitution of the ADP glucose Transporter

The solubilized ADP-glucose transporter was reconstituted into liposomes using the procedure described by Tetlow *et al.* (1996) to form proteoliposomes. The transporter was orientated such that it pumped ADP-Glucose out of the liposome (i.e the reverse orientation to that found in the amyloplast where ADP-Glucose is taken into the amyloplast). Transport of ATP, ADP glucose, AMP and ADP was then determined (also using the procedure described by Tetlow *et al.* (1996)).

Transport of various radiolabelled substrates (1mM) into proteoliposomes (containing the purified protein) was measured when the proteoliposomes were preloaded with various counter-exchange substrates (10mM). Table 1 shows that the transport rate was highest when ADP or AMP were used as substrates and ADP-Glucose was used as the counter-exchange substrate. This illustrates that the isolated protein has ADP-glucose transport activity and furthermore acts as an antiporter transporting ADP-glucose in exchange for ADP and AMP.

TABLE 1

SUBSTRATE (1mM)	COUNTER-EXCHANGE	TRANSPORT RATE
	SUBSTRATE (10mM)	(nmol/min/mg protein)
ATP	Buffer	33
ADP-Glucose	Buffer	32
ADP	ADP-Glucose	99
AMP	ADP-Glucose	109

References

- Tetlow *et al.* (1993) *Planta* 189: p597-600
Tetlow *et al.* (1994) *Planta* 194: p454-460
Tetlow *et al.* (1996) *Biochem. J.* 319: p717-723.

CLAIMS

1. An ADP-Glucose transporter protein; or a modification or fragment thereof capable of ADPG transport activity comprising at least one amino acid sequence selected from the group of:

- (i) SMPLNAAVKM;
- (ii) GAXXXETAWACGXA;
- (iii) PYNEAYQOXG; and
- (iv) NFRYTNFAXF.

2. The ADP-Glucose transporter protein according to claim 1 wherein the protein comprises each of the amino acid sequences (i) - (iv).

3. The ADP-Glucose transporter protein according to claim 1 or 2 wherein the protein has a molecular weight of about 38kDa.

4. A DNA molecule encoding for a protein which directly or indirectly modulates ADP-glucose transporter protein activity, said DNA molecule being capable of being transcribed to lead to the expression of said protein.

5. The DNA molecule according to claim 4 encoding a protein according to any one of claims 1-3.

6. A plant cell transformed with the DNA molecule according to claim 4 or 5.

7. A method of regulating starch production from a plant, plant tissue or plant cell comprising modulating activity of the amyloplast membrane ADP-Glucose transporter protein.

8. The method according to claim 7 wherein activity of the ADP-Glucose transporter is increased.
9. The method according to claim 8 wherein the yield of starch is increased relative to an unmodulated plant, plant tissue or plant cell.
10. The method according to claim 8 or 9 wherein starch is produced with a higher proportion of amylose than found in starch from an unmodulated plant, plant tissue or plant cell.
11. The method according to claim 10 wherein starch is produced with increased viscosity and gel strength relative to starch from an unmodulated plant, plant tissue or plant cell.
12. The method according to claim 10 wherein the starch is incorporated in a baked food which goes stale less quickly than baked food containing starch derived from an unmodulated plant, plant tissue or plant cell.
13. The method according to any one of claims 8 - 12 wherein the plant, plant tissue or plant cell is transformed with the DNA molecule according to claims 4 or 5.
14. The method according to claim 7 wherein the activity of the ADP-Glucose transporter is decreased.
15. The method according to claim 14 wherein starch is produced with a higher proportion of amylopectin than found in starch from an unmodulated plant, plant tissue or plant cell.
16. The method according to claim 15 wherein "waxy" starches are produced for use as thickening agents in food and coatings.

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17. An ADP-Glucose transporter protein obtainable by the protocol of Example 1.



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